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Development of methods for quantification and classification of gelatin in capsule shell using chemometric analysis of FTIR spectroscopic data

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Abstract

Capsule shell from animal source (bovine or porcine gelatin) is a problem for the follower of different religions and vegetarian. In that case, vegetable capsule shell could be a solution. In this research, we proposed a simple and cost-effective technique for detection of gelatin in vegetable capsule shell and for classification of capsule shell by source, based on Chemometric techniques with FTIR spectroscopic data. Partial Least-Square Regression (PLSR) models were developed and their efficiencies were assessed with spectroscopic data of range of 4000-700 cm^{-1}. PLSR shows very good prediction efficiency (R^2= 98%) for both vegetable capsule shell and gelatin. In addition, Soft Independent Modeling by Class Analogy (SIMCA) classification method were developed and assessed with spectral data of capsule shells from vegetable and animal sources. Results prove that FTIR spectroscopy in combination with chemometric method can be used for the classification of capsule shell by source and quantification of gelatin in vegetable capsule shell to ensure their authenticity.

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Introduction

Capsule is a solid and soluble container of drug in hard or soft for their easy intake. The capsule shells are produced from animal or vegetable sources. The capsules shells used today for drug administration are mainly made of Gelatin, and it is the most studied element in Halal researches. The most common source of commercial gelatin is mammalian (bovine and mostly porcine) bone and hide (Shabani *et al*., 2015). It is made by fractional hydrolysis of collagen extracted from the skin, bones and connective tissues of animals. So, due to cultural and religious beliefs consumers are concern about gelatin sources. For instance, Muslims and Jews refuse porcine originated food derivatives, beef based food derivatives are prohibited in Hinduism, Chinese conventional medicine made of gelatin from donkey skin are used for treatment of some sickness (Nemati *et al*., 2004) and vegetarians keep away from animal based products. Moreover, the percentage of gelatin production in the world in 2006, still dominated by pig gelatin, with 45.8% the materials is coming from pigskin and 28.4% from cowhide (Phillips, 2009). In this case, capsule shell from vegetable source can be the best alternative to the gelatin based capsule shell.

Thesedays, vegetable capsules are perfect alternative which might be replacing the intake of gelatin or non-vegetable capsules. Hydroxy propyl methyl cellulose (HPMC) is mainly used as alternative for gelatin in the preparation of vegetable capsule shell. It is prepared by synthetic alteration of the naturally occurring polymer cellulose.

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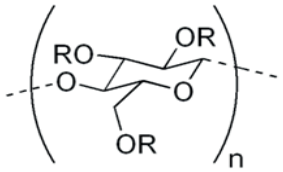

R = H or CH_3 or $CH_2CH(OH)CH_3$

Fig. 1. Structure of HPMC

Many methods have been developed so far for gelatin source confirmation, such as: spectroscopic method (Hashim *et al*., 2010; Hermanto and Fatimah, 2013), immunochemical method (Tukiran *et al*., 2015, 2016), nucleic acid based method (Cai *et al*., 2012; Malik *et al*., 2016; Mutalib *et al*., 2015; Shabani *et al*., 2015; Sudjadi *et al*., 2015), mass spectrometric method HPLC/MS (Yilmaz *et al*., 2013; Zhang *et al*., 2008; Zhang *et al*., 2009), by amino acid analysis using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate as derivatization reagent (Azilawati *et al*., 2015), electrophoretic analysis (Azira *et al.*, 2014) and chemisorption (Hidaka and Liu, 2003). These methods need much time and capital intensive equipment with vast running cost, involve complex separation methods and require the use of different chemicals that are harmful to the environment. These drawbacks can be overcome by using multivariate analysis of spectroscopic data based chemometric methods which is less time consuming, limited use of chemical, straightforward and simple (Israt *et al*., 2016; Hassan, *et al*., 2018; Uddin *et al*., 2019). However, development of methods for quantification of gelatin in vegetable capsule shell and for classification of capsule shell as from vegetable or animal sources by chemometric analysis of FTIR spectroscopic data is a novel one through this study.

Materials and methods

Reagents, chemicals and samples

Empty vegetable capsule shell and gelatin capsule shell were collected from ACG and Capsugel company and pure gelatin (bovine) was collected from ACI Pharmaceuticals. Four different local commercial samples were purchased from the local shops. Vegetable capsule shell and gelatin (bovine) with highest purity were used for spiking.

Preparation of standard solutions

Total 32 standard solutions containing mixture of gelatin and vegetable capsule shell solution were prepared for the development of chemometric calibration model. Gelatin solutions were mixed with vegetable capsule shell solution in different concentration by following Orthogonal Experimental Design (OED) (Table I) using the software "SPSS" (version 22.0). Besides, standard samples from 2 different sources (animal and vegetable) were taken with various concentration following OED for developing classification method (Table II).

Table I. Composition of calibration samples through orthogonal experimental design

Sample ID	Vegetable capsule shell (mg/mL)	Gelatin (mg/mL)	Sample ID	Vegetable capsule shell (mg/mL)	Gelatin (mg/mL)
101	10	0.0	117	2.0	8.0
102	9.5	0.5	118	1.5	8.5
103	9.0	1.0	119	1.0	9.0
104	8.5	1.5	120	0.5	9.5
105	8.0	2.0	121	0.0	10
106	7.5	2.5	201	7.0	0.0
107	7.0	3.0	202	6.3	0.75
108	6.5	3.5	203	5.6	1.5
109	6.0	4.0	204	4.9	2.25
110	5.5	4.5	205	4.2	3.0
111	5.0	5.0	206	3.5	3.75
112	4.5	5.5	207	2.8	4.5
113	4.0	6.0	208	2.1	5.25
114	3.5	6.5	209	1.4	6.0
115	3.0	7.0	210	0.7	6.75
116	2.5	7.5	211	00	7.5

Table II. Experimental design for classification models

Sample ID	Source	Concentration (mg/mL)	Sample ID	Source	Concentration (mg/mL)
501	Vegetable	10	601	Animal (Bovine)	10
502	Vegetable	9.5	602	Animal (Bovine)	9.5
503	Vegetable	9.025	603	Animal (Bovine)	9.025
504	Vegetable	8.574	604	Animal (Bovine)	8.574
505	Vegetable	8.145	605	Animal (Bovine)	8.145
506	Vegetable	7.738	606	Animal (Bovine)	7.738
507	Vegetable	7.351	607	Animal (Bovine)	7.351
508	Vegetable	6.983	608	Animal (Bovine)	6.983
509	Vegetable	6.634	609	Animal (Bovine)	6.634
5091	Vegetable	6.464	6091	Animal (Bovine)	6.464
510	Vegetable	6.133	610	Animal (Bovine)	6.133
5101	Vegetable	5.818	6101	Animal (Bovine)	5.818

Spectral data acquisition

Fourier Transform Infrared (FTIR) spectrophotometer (Model-IR Prestige 21, Shimadzu, JAPAN) was used for measuring the absorbance of different samples. Spectral data of vegetable capsule shell, gelatin and standard samples were collected using FTIR spectrophotometer in the wave number range of 4000-700 cm^{-1} at a resolution of $1cm^{-1}$. Obtained spectroscopic data were processed and the models were developed by a licensed copy of CAMO the Unscrambler (Ver. 10.5).

Chemometric model development

In recent years, multivariate analysis of spectral data based chemometric strategies appear to be the techniques showing the best execution for method development in analytical chemistry. Each spectrum contains huge number of absorbance value for each wave point. Every wave point or data point is considered as spectroscopic variable. These variables are huge in number and are mutually correlated. So, Ordinary Least Square (OLS) method cannot be used as there is a problem of singularity. Therefore, predictive efficiencies of the most popular calibration chemometric technique Partial Least Square Regression (PLSR) (Martens and Naes, 1996; Wold *et al.,* 2001; Brereton, 2000; Naes *et al.,* 2002) has been assessed in this study. For classification model development, Soft Independent Modeling by Class Analogies (SIMCA) (Wold, 1976; Wold and Sjöström, 1977) has been used. The fitness of the models were evaluated by coefficient of multiple determination (R^2) and root means square error (RMSE).

Partial Least Square Regression (PLSR)

PLS regression finds components from independent variables (X) that best predict the dependent variable (Y). Specifically, PLS regression searches for a set of components (called latent vectors) that performs a simultaneous decomposition of X and Y with the constraint that these components explain as much as possible of the covariance between X and Y. This step generalizes PCA. It is followed by a regression step where the latent vectors obtained from X are used to predict Y. PLS regression decomposes both X and Y as a product of a common set of orthogonal factors and a set of specific loadings (Wold *et al.* 2001; Kramer, 1998).

Cross-validation

In order to evaluate calibration efficiency of the developed model, 6-fold cross-validation techniques have been used to minimize the risks of over-fitting of the developed models. Cross-validation, sometimes called rotation estimation or out-of-sample testing, is any of various similar model validation techniques for assessing how the results of a statistical analysis will generalize to an independent data set. Cross-validation is a resampling method that uses different portions of the data to test and train a model on different iterations. It is mainly used in settings where the goal is prediction, and one wants to estimate how accurately a predictive model will perform in practice (Varmuza and Filzmoser, 2008). In summary, cross-validation combines (averages) measures of fitness in prediction to derive a more accurate estimate of model prediction performance. 6-fold cross-validation technique is popularly used to test the model efficiency, and hence it has been used in the present study. Six-fold cross-validation datasets are produced through

CAMO the Unscrambler software also to find the stability of the developed models when they will be used in real situation.

Results and discussion

Spectra of vegetable capsule shell, gelatin and standard solution which is the mixture of both, are shown in Fig. 2. It can be seen from the figure that the spectra of vegetable capsule shell, gelatin and the mixture have overlapped significantly. The quantification of the adulterants in the mixture by traditional univariate calibration method is interrupted by the overlapping spectra. So, multivariate calibration technique were carried out.

The score plot in the Fig. 3 shows the plot of the first principal component (PC1) against the second one (PC2) for the calibration mixtures. From Principal Component Analysis (PCA) it is shown that the first principal component (PC1) expresses 93% of the variation whereas PC2 express 4% of the total variation. So the sum of 97% is good enough for an excellent representation of the dimensional variable spaces in the two dimensional projection.

Fig. 4 represents the influence plot. This plot is used before model development to identify if there is any outlier in the data. Outliers are cases that do not correspond to the model fitted to the bulk of the data. Outliers may be generated by typing errors, interface errors, file transfer, sensor

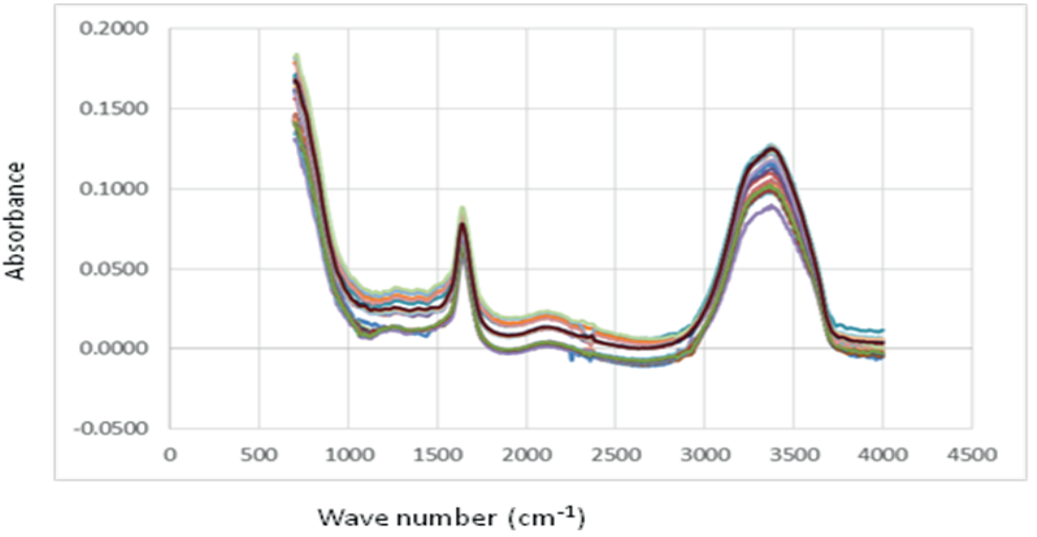

Fig. 2. Spectral (FTIR) presentation of absorbance against wavenumber

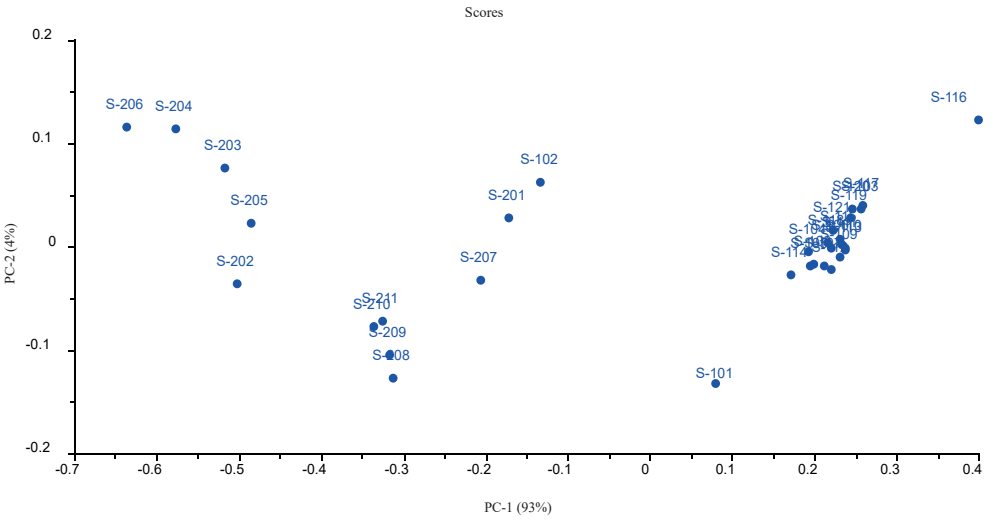

Fig. 3. Association between samples (score plot) or map of samples

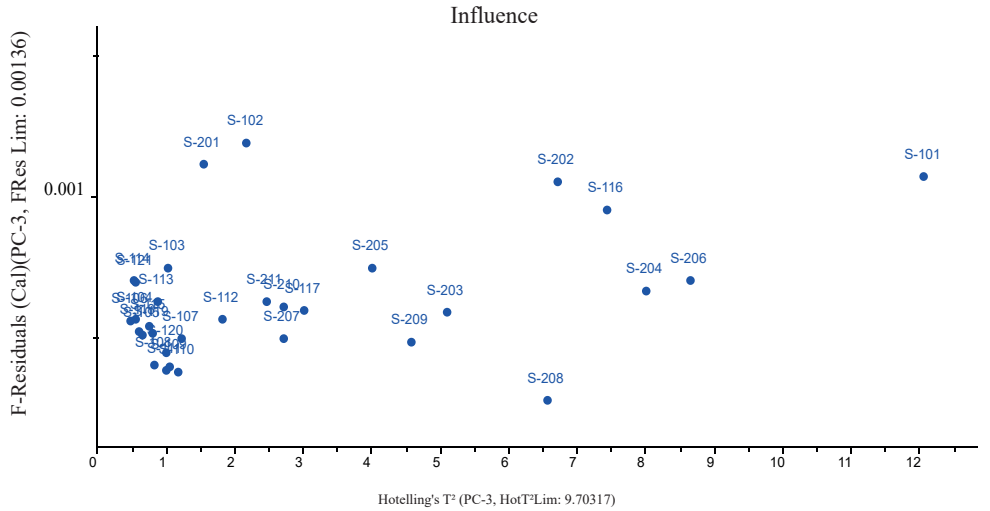

Fig. 4. Influence plot

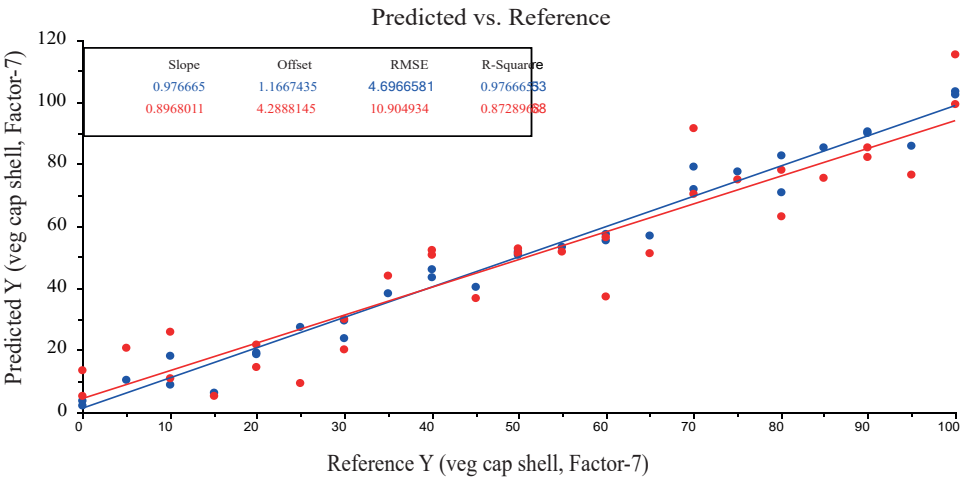

Fig. 5. Predicted vs. Reference plot for vegetable capsule shell

malfunctions and fouling, poor sensor calibration, error in sample preparation and cross contamination. PCA is very sensitive to outliers and can lead to misleading results when outliers are present. From the influence plot (Fig. 4), we can see that sample ID-101 is considered as a outlier, and this sample has been excluded from model development process in the study.

Spectral values and concentrations of the mixture samples obtained from the experimental design are considered as data matrix for the study. 75% mixture solutions were used for the development of the model and remaining 25% mixture solutions were used for the validation of the developed model.

Prediction model

Performance of Partial Least Square Regression (PLSR) models for predicting vegetable and gelatin portion in capsule shell have been developed. The developed PLSR models are assessed by different parameters to see the effectiveness of the model for predictive purpose. Here two model efficiency parameters such as Root Mean Square Error (RMSE) and Coefficient of Multiple Determination (R^2) have been considered.

Here, for vegetable capsule shell, the RMSE value of PLSR is 4.7 which is low, and R^2 is 97.67% which is relatively high. Therefore, the model is a good one to predict the authenticity of vegetable capsule shell. At the same time the model is reasonably stable as R^2 is 87.23% for model validation dataset.

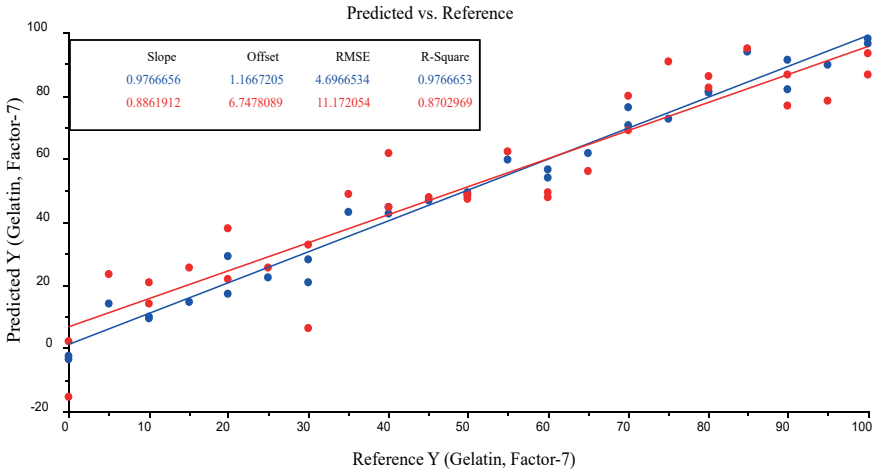

Fig. 6. Predicted vs. Reference plot for gelatin

Table III. Predicted values of vegetable ingredient and gelatin in commercial samples (CS)

Sample ID	Ingredients of Vegetable in capsule shell s (mg/100mL)	Gelatin in capsule shell s (mg/100mL)
CS 01	33.1342	66.8655
CS 02	67.3931	32.6075
CS 03	98.1841	1.8158
CS 04	80.6639	19.3360

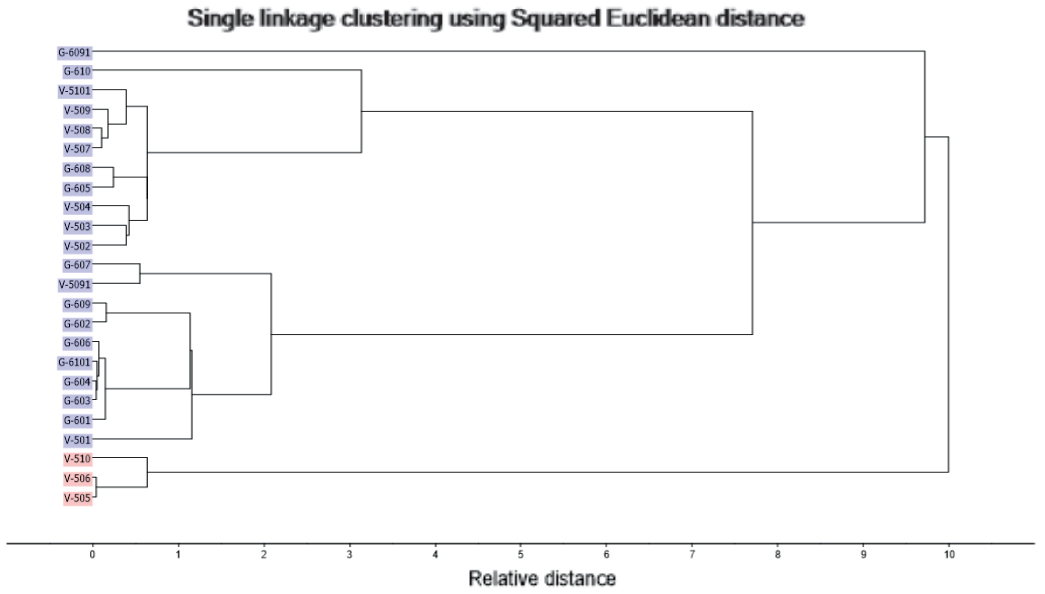

Fig. 7. Dendogram for clustering of capsule shell by source

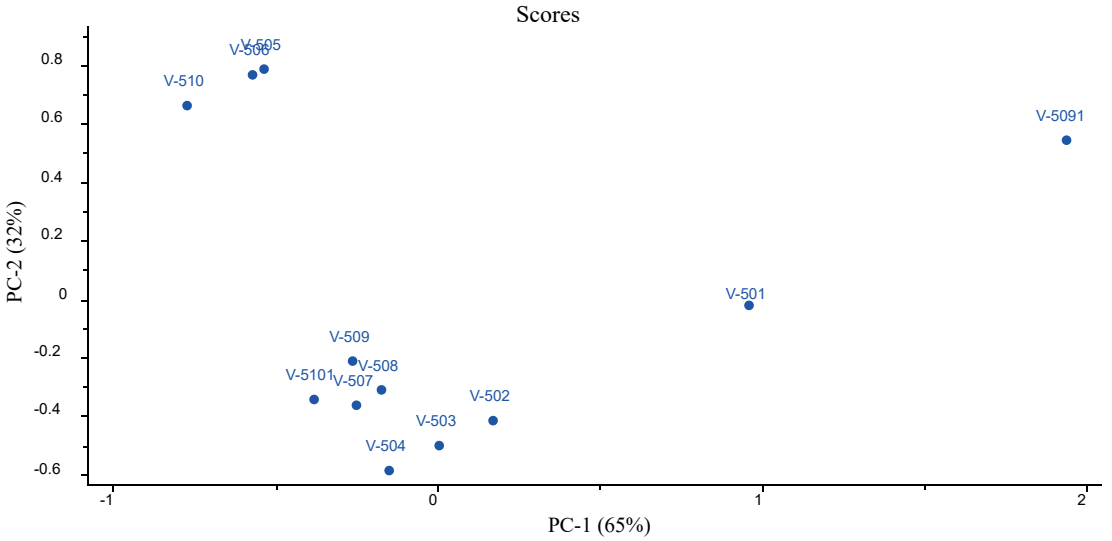

Fig. 8. Association between samples (score plot) or map of samples vegetable source

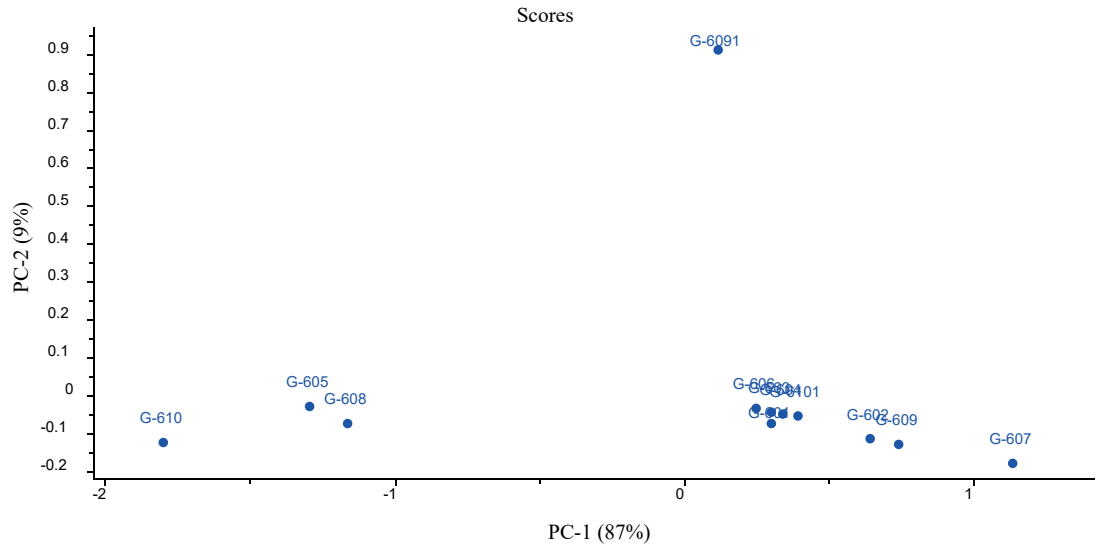

Fig. 9. Association between samples (score plot) or map of samples animal source (Bovine)

Here, for gelatin, very low RMSE value of PLSR which is 4.7 which is low. Another model efficiency parameter R^2 is 97.67% which is relatively high. Therefore, the model is a good one to predict gelatin in vegetable capsule shell. This model is also very stable as R^2 is 87.03% with model validation dataset. As the RMSE value is low and R^2 value is high for PLSR model of both vegetable capsule shell and gelatin, so no preprocessing technique for the spectral data, have been applied in this study.

Predicted Results

The commercial samples are then run into the model to predict the amount of vegetable ingredient and gelatin in capsule shells. The predicted data is given in table III.

From the table III, it is evident that these commercial samples contain gelatin even though the capsule shells are claimed from vegetable source commercially, but they should be authentic and should not be mixed with gelatin which is from animal source.

Table IV. Classification of capsule shells sample by source (considering 1% and 5% error)

Sample ID	1% error		5% error	
	PCA for Vegetable source	PCA for Gelatin source	PCA for Vegetable source	PCA for Gelatin source
V-501	*		*	
V-502	*		*	
V-503	*		*	
V-504	*		*	
V-505	*		*	
V-506	*		*	
V-507	*		*	
V-508	*		*	
V-509	*		*	
V-5091	*		*	
V-510	*		*	
V-5101	*		*	
G-601		*		*
G-602		*		*
G-603		*		*
G-604		*		*
G-605	*	*	*	*
G-606		*		*
G-607		*		*
G-608	*	*	*	*
G-609	*	*		*
G-6091		*		*
G-610		*		*
G-6101		*		*

Clustering the samples

In order to get a preliminary idea before developing classification models based on their sources, either vegetable source or animal source, samples were clustered by using the technique of drawing Dendogram.

Here, Normalized Euclidean distances and the Ward's method were used to get dendrogram (Einax *et al*., 1997). At relative distance 8, cluster I is formed G-6091, cluster II by G-610,V-5101,V-509,V-508,V-507,G-608,G-605,V-504,V-503,V-502, cluster III by G-607,V-5091,G-609, G-602, G-606,G-6101,G-604,G-603,G-601 and finally cluster IV by V-501,V-510,V-506.

Classification Model

For classification of capsule shells by their sources using Soft independent Modeling by Class Analogy (SIMCA) method, PCA model was developed for each class. Here is the graphical representation of PCA model of the sample set for classification of vegetable based capsule shell and gelatin based capsule shell.

From the Principal Component Analysis (PCA) of samples from vegetable source (Sample ID: 501-5101), it is found that the first principal component (PC1) expresses 65% of the variation whereas PC2 expresses 32% of the total variation. First two PCs express 97% of total variation which is good enough for an excellent representation of the 12 dimensional variable spaces in the two dimensional projection.

From the Principal Component Analysis (PCA) of samples from animal source (Sample ID: 601-6101), it is noticed that the first principal component (PC1) express 87% of the variation whereas PC2 express 9% of the total variation. So the sum of 96% is good enough for a very good representation of SIMCA model.

The table IV was generated in CAMO the Unscrambler Software by SIMCA classification models considering 1% and 5% error. Here, in case of 1% error consideration, 100% of the samples from vegetable source (Sample Id: 501 to 5101) are correctly classified by the developed model. On the contrary, 100% of the sample from gelatin source (Sample Id: 601 to 6101) are rightly classified, but the sample 605, 608, 609 are in the both group (12.5%) where they should be

in the gelatin source group by the developed SIMCA model considering 1% error.

However, when 5% error was considered, all samples from vegetable source are classified 100% accurately; however, only two samples out of 24 (8%) samples from gelatin source are misclassified as from vegetable source. Further, if we consider 8% error, the SIMCA model can separate all the sample by source perfectly, ie, 100% accurately.

Conclusion

From this study, we can conclude that PLSR model with FTIR spectroscopic data can be used to quantify the amount of vegetable and gelatin in mixed capsule shell (R^2=97.67%). The proposed technique based on multivariate analysis of FTIR spectroscopic data requires less chemical waste, quick, non-dangerous, straightforward and simple to utilize for the detection of such adulterant.

Moreover, Soft Independent Modeling by Class Analogies (SIMCA) also based with FTIR spectroscopic data analysis. Here, 100% of the samples from vegetable source are correctly classified by the developed model. On the contrary, 100% of the sample from gelatin source are rightly classified, but three falls under both groups (12.5%) when we consider 1% error. The rate of misclassification become 8% if 5 % error is considered. However, if the error is 8%, then 100% samples are correctly classified by their source with the developed SIMCA model.

For the prediction and classification of capsule shell from gelatin or vegetable sources, these two proposed method could be used for quality control or routine analysis of pharmaceuticals with minimum testing time and cost compared to traditional methods.

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